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Isolation and characterization of lipids strictly associated to PSII complexes: Focus on cardiolipin structural and functional role

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Abstract

In this work, lipid extracts from spinach membrane fragments enriched in Photosystem II (PSII) and from spinach PSII dimers were analyzed, by means of Thin Layer Chromatography (TLC) and Electro-Spray Ionization Mass Spectrometry. Cardiolipin found in association with PSII was isolated and purified by preparative TLC, then characterized by mass and mass–mass analyses. Cardiolipin structures with four unsaturated C18 acyl chains and variable saturation degrees were evidenced. Structural and functional effects of different phospholipids on PSII complexes were investigated by Fluorescence, Resonance Light Scattering and Oxygen Evolution Rate measurements. An increment of PSII thermal stability was observed in the presence of cardiolipin and phosphatidylglycerol.

Keywords: Photosystem II; Cardiolipin; Electro-Spray Ionization Mass Spectrometry; Resonance Light Scattering; Fluorescence Emission; Oxygen Evolution Rate

1. Introduction

Photosynthesis is a very important biochemical process which synthesizes carbohydrates starting from carbon dioxide and an electron donor (water in higher plants): this process is thermodynamically unfavourable, and this is the reason why photosynthetic organisms use light energy. In higher plants, Photosystem II (PSII) is one of the multisubunit proteic membrane complexes which are involved in the "light reactions": it converts light energy into the electrochemical energy required for the water oxidation. It is embedded in the lipid matrix of the thylakoid membrane of the chloroplasts and it strictly cooperates with Photosystem I (PSI) and the cytochrome b_6f complex [1–3].

The isolated dimeric form of PSII complexes shows higher activity than its monomeric counterpart, suggesting that the dimer represents an in *vivo* aggregation state of PSII which is more efficient [4–7]. This implies that investigations into the factors involved in the aggregation processes are interesting for biotechnological applications of PSII in environmental and energetic fields, as well as for a more detailed knowledge of photosynthetic complexes.

In particular, lipid environment is very critical in fixing the biophysical and biochemical properties of numerous intrinsic proteins. It is widely accepted that specific protein–lipid interactions are very important for the structural and functional integrity of many prokaryotic and eukaryotic membrane

Abbreviations: CL, Cardiolipin; Chl, Chlorophyll; DCBQ, 2,5-dichloro-p-benzoquinone; DM, n-dodecyl-β-D-maltoside; ESI-MS, Electro-Spray Ionization Mass Spectrometry; ESI-MS-MS, Electro-Spray Ionization Mass–Mass Spectrometry; LHCI, Light Harvesting Complex I; LHCII, Light Harvesting Complex II; MNCB, aqueous buffer containing [2-N-morpholine]ethanesulphonic acid (25 mM), NaCl (10 mM), $CaCl_2$ (5 mM) and $NaHCO_3$ (10 mM); OG, n-octyl-β-D-glucopyranoside; OER, Oxygen Evolution Rate; PA, Phosphatidic acid; PG, Phosphatidylglycerol; PI, Phosphatidylinositol; PSI, Photosystem I; PSII, Photosystem II; Qq-TOF, Quadrupole quadrupole time of flight; RC, Reaction Centre; RLS, Resonance Light Scattering; SQDG, Sulfoquinovosyldiacylglycerol; TLC, Thin Layer Chromatography

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proteins [8]. Specific roles played by phosphatidylglycerol (PG) in the electron transport at the Q_B-binding site in PSII complexes and in its dimerization have been reported [4,9]; furthermore, evidence of an enrichment of cardiolipin (CL) in the purified PSII complexes has been recently documented by our group [10]. CL was found to be enriched throughout the purification of PSII complexes; in particular, dimers were found to contain two times more CL per RC than their monomeric counterparts.

Among anionic phospholipids, CL (known also as diphosphatidylglycerol) is unique, because of its dimeric structure with four acyl chains and two negative charges. Typically it is placed in membranes which produce an electrochemical potential for substrate transport and ATP synthesis. In animal tissues, CL contains almost exclusively 18-carbon fatty acids (C18), and 80% of these are typically linoleic acid and this also appears to be true for the higher plants [11,12].

Although several studies have shown a strict requirement for CL by mitochondrial and bacterial bioenergetic complexes, the precise functions of CL have not been yet clarified. It is known that the function of respiratory chain complexes can be affected by changes in concentration of CL or its fatty acid composition [13]. Certainly, all proteins involved in the oxidative phosphorylation interact with CL; for example, the respiratory protein cytochrome *c* oxidase is inactivated by removing of the CL [14]. Moreover, it is widely known that it is possible to preserve the stability and functions of mitochondrial carrier proteins by adding exogenous CL during the protein isolation and purification procedures [15]. Recently, X-ray structures have evidenced a specific binding of CL on the cytoplasmic side of the reaction centre (RC) from *Rhodobacter sphaeroides*, in contact with all three subunits [16–18]. Fyfe et al. have also highlighted the relevance of CL on the RC of *R. sphaeroides* thermal stability [18], while Maròti et al. have shown that CL can affect the quinone Q_A free energy, by interactions at other specific lipid binding sites [19]. Moreover, data in the literature point out the presence of two different CL species in *R. sphaeroides*, showing that CL content increases under osmotic stress conditions; this last phenomenon has been also observed in different microorganisms [8,20]: it has been suggested that CL might contribute to proton active transport [21] and sustain the cell bioenergetic system under osmotic shock [20].

In higher plants and in particular in chloroplasts, understanding of the roles of CL is still quite poor; therefore, in this work, the attention has been directed to the lipid composition analysis of membrane fraction increasingly enriched in PSII complexes. In particular, CL associated to PSII has been isolated and purified by preparative Thin Layer Chromatography (TLC); lipid extracts and isolated lipid species have been analyzed by TLC and Electrospray Ionization Mass Spectrometry (ESI-MS). Furthermore, the influence of lipids on structural and functional properties of PSII complexes have been investigated by means of Fluorescence, Resonance Light Scattering (RLS) and Oxygen Evolution Rate (OER) measurements.

2. Materials and methods

2.1. Materials

Triton X-100, n-dodecyl-β-D-maltoside (DM), n-octyl-β-D-glucopyranoside (OG), chloroform (99.8%), molibdenum blue spray reagent (1.3%) and lipid standards were purchased from Sigma; methanol (99.8%) was purchased from J. T. Baker; acetone (99.8%) and sulphuric acid (96%) were purchased from C. Erba; acetic acid (96%) was purchased from Riedel-de Haën; 2,5-dichloro-p-benzoquinone was purchased from Kodak; TLC plates (10×20 cm) were from Merck.

2.2. Isolation of chloroplasts, thylakoids, membrane fractions enriched in PSII, PSII core monomers and dimers

Chloroplasts, thylakoids and membrane fractions enriched in PSII were isolated from market spinach leaves according to Hankamer's procedure [22–25]. Chloroplasts were obtained from spinach leaves using differential centrifugation and thylakoids from chloroplasts by their rupture in a hypotonic solution. Thylakoids were solubilized with Triton X-100 in order to extract PSII-enriched membranes (BBYs). These last membranes were solubilized with OG to detach the LHCII proteins from the PSII core (OG-core complexes). OG-core complexes were then diluted with an aqueous buffer (MNCB) containing [2-N-morpholine]ethane-sulphonic acid (25 mM), NaCl (10 mM), $CaCl_2$ (5 mM) and $NaHCO_3$ (10 mM), and DM was added to the solution, in order to obtain a chlorophyll (Chl) concentration of 0.5 mg/ml and a 25-mM DM concentration. Sucrose gradients, supplemented with DM (0.03%, w/v), were employed to obtain oxygen evolving PSII core monomers and dimers which lack the 23- and 17-kDa extrinsic proteins and the CP29, CP26, CP24 chorophyll binding proteins. The concentration of PSII complex samples was estimated as Chl mg/ml [26].

2.3. Extraction and purification of lipids from PSII preparations

Polar lipids and non-polar pigments (chlorophylls and carotenoids) were extracted from BBYs and from PSII dimers prepared from the same batch of spinach, according to the Bligh and Dyer standard method [27]. Before the extraction, PSII preparations were concentrated using Amicon Centricon 100 concentrator and subsequently resuspended in MNCB at final concentration of 1 mg of Chl/ml. Briefly, 6 ml of methanol–chloroform 2:1 (v/v) was added to 1.6 ml of suspension of BBY or PSII dimer fraction in aqueous MNCB buffer. The mixture was shaken for 15 min, then centrifuged and the supernatants transferred by means of a Pasteur pipette to another tube. The residual precipitated material was then resuspended in 7.6 ml of methanol–chloroform–water 2:1:0.8 (v/v) and the mixture was again shaken for 15 min and centrifuged. The combined extracts were diluted with 8 ml of chloroform–0.2 M KCl 1:1 (v/v) in order to have a final mixture of methanol–chloroform–water 1:1:0.9 (v/v). Then the phase separation was obtained by centrifugation; the lower chloroform phase was withdrawn, and about 2 ml of chloroform was added to chloroform–water phase to optimize the lipid recovery. The combined chloroform phases were dried in a rotatory evaporator and the lipids and pigments obtained were redissolved in chloroform/methanol (1:1, v/v) at final concentration of 10 μg/μl and stored at −20 °C. Since highly polar lipids (and in particular sulphated polyglycolipids) may partition into the methanol–water phase we found that in the presence of salt this inconvenience can be avoided and in particular that PG and cardiolipin are quantitatively collected in the lower chloroform phase. The absence of lipids in the methanol–water phase was verified by means of TLC analysis (data not shown).

2.4. Removal of pigments

In order to separate neutral (including pigments) from polar lipids, BBY or PSII dimer lipid extracts were precipitated in ice-cold acetone as follows.

20 volumes of ice-cold acetone were added to a volume of concentrated lipids in chloroform, mixed and then stored at −20 °C overnight. Phospholipids were collected by centrifugation and the supernatants containing neutral lipids

and pigments were removed; the precipitate was washed two times with a small volume of ice-cold acetone and centrifuged as above.

It should be observed that the polar lipid extract obtained after acetone precipitation procedure retains the qualitative composition of the total lipid extract. Therefore, the extracts obtained by the ice-cold precipitation method were found to be suitable for identification of lipids present in the BBY or PSII dimer lipid extracts by mass spectrometry analyses, and for CL isolation.

2.5. Analysis of PSII lipid components by TLC

The lipid mixture extracted from PSII membrane preparations was resolved into its components by TLC for qualitative analysis. The selected amounts of each sample (10 μg/μl) were dispensed onto Silica Gel 60 TLC plates. Unless otherwise specified, the lipid components were separated by using a chloroform, methanol, acetic acid and water (75:13:9:3, v/v) mobile phase. The separated glycolipids and phospholipids were detected by spraying the TLC plates with an acid aqueous solution (5% sulphuric acid) and incubating them at 120 °C for 5 min. Only for the TLC reported in panel "b" of Fig. 1 (lane 3) the mobile phase was chloroform, methanol, water (65:25:4, v/v).

Endogenous cardiolipin was isolated from the BBY lipid extract by preparative TLC; the silica corresponding to the band of CL, identified by using an authentic standard, was scraped out. The CL was extracted from the silica with chloroform/methanol (1:1,v/v) five times; after drying the organic solvents in a rotatory evaporator, CL was dissolved (10 μg/μl) in chloroform/methanol (1:1, v/v) and stored at −20 °C.

2.6. Mass spectrometry

All mass spectrometry determinations were carried out in flow injection analysis with dried lipid samples that were dissolved in chloroform/methanol (1:1, v/v). 2 μl samples, injected via a 10-μl loop, were transferred into the MS electrospray interface with a flow rate of 0.1 ml/min of chloroform/methanol (1:1, v/v) delivered by a Perkin-Elmer 200 chromatographic system.

Low-resolution mass spectra were obtained with an API 165 mass spectrometer (Applied Biosystem/MSD Sciex, Canada) equipped with a turbo ion spray interface. Interface conditions for the detection of negative ions were as follows: nebulizer gas (air)=1.2 l/min, curtain gas (nitrogen)=1 l/min, needle voltage=−5000 V, declustering potential=−150 V and focusing potential=−200 V.

High-resolution mass spectra were obtained with a QSTAR hybrid Qq-TOF mass spectrometer (Applied Biosystem/MSD Sciex, Canada) equipped with a turbo ion spray interface. Interface conditions for the detection of negative ions were as follows: nebulizer gas (air)=1.2 l/min, curtain gas (nitrogen)=1 l/min, needle voltage=−4500 V, declustering potential=−50 V, focusing potential=−300 V. Errors associated with such determinations were within 40 ppm. MS-MS measurements were carried out by fragmenting the target ions at proper collision energy (usually 35 eV).

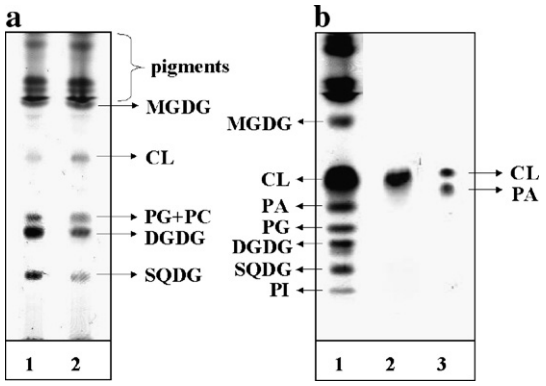

Fig. 1. TLC analyses of lipid extracts. Panel "a": lane 1, BBY lipid extract; lane 2, PSII dimer lipid extract. 50 μg of lipids of each sample were loaded and the plate stained by 5% H_2SO_4 solution. Panel "b": preparative TLC. Lane 1, BBY lipid extract (200 μg) after impoverishment in pigments; lane 2, band of CL extract obtained from spinach BBY fragments; lane 3, CL extract in a different chromatographic system. (See paragragh 2.5.)

2.7. Steady-state fluorescence and Resonance Light Scattering measurements

Fluorescence measurements were carried out using a Varian Cary Eclipse spectrofluorimeter. RLS spectra were obtained on the same spectrofluorimeter, according to a synchronous scan protocol with a right angle geometry [28].

For the experiments, 1 cm path length quartz cells were used; for highly scattering samples, neutral density filters were set in the excitation path. The RLS spectra were corrected by subtracting the RLS of lipids in buffer solution. In this study the concentration of Chl used for fluorescence and RLS measurements was 0.015 mg/ml. In the experiments PG and CL were added to PSII solutions: the obtained lipid/PSII ratio (mol/mol) was about 100, in accordance with the experimental observations by Kruse et al. [4].

2.8. Oxygen Evolution Rate measurements

Oxygen evolution rates were obtained by means of a composite oxygraphic device by Rank Brothers. Experiments were conducted at room temperature, by adding 0.04 mmol of the oxidizing reagent 2,5-dichloro-p-benzoquinone (DCBQ) per mg of chlorophyll to samples and illuminating them with an optical fiber with a lamp at 150 Watt (lamp temperature 3 200 K). PSII samples were at concentration of 0.015 mg of Chl/ml. In the experiments exogenous PG and CL were added to PSII solutions: the obtained lipid/PSII ratio (mol/mol) was about 100, in accordance with the experimental observations by Kruse et al. [4].

Values of about 400 μmol O_2/h mg chl were obtained for freshly prepared PSII complexes.

2.9. Statistical analysis

The GraphPad InStat software (Sigma, St. Louis, MO) was used to process the data by analysis of variance (ANOVA) to indicate statistically significant differences between means (one-way ANOVA with post-hoc Tukey test, $p<0.05$).

All reported data represent mean values (standard deviation obtained from three replicates).

3. Results and discussion

Fig. 1 reports the TLC analysis of the lipid extracts from different samples: in panel "a" of the figure, the analysis is relevant to the total lipid extract of the BBYs and PSII dimer, respectively. It can be observed that the lipid composition is preserved throughout the purification, and the fractions differ only in the relative lipid percentage. In agreement with a previous report [10], the CL content was found to be higher in PSII dimer than in BBY lipid extract.

Nevertheless, our results are in contrast with those obtained by Sakurai et al., which could not find evidence for the presence of CL among lipids associated with PSII systems isolated from spinach and rice by both ion exchange chromatography and size-exclusion chromatography [9]. This contradictory result might be due to the differences in the procedures used for purification of PSII complexes, resulting in a different extent of lipid removal from the protein complexes.

In order to avoid the interference of pigments in the ESI-MS analyses of the lipid extracts, BBY or PSII dimer lipid extracts were precipitated in ice-cold acetone to remove most of the green pigments.

The first lane of the panel "b" of Fig. 1 reports the TLC analysis of BBY lipid extract after removal of most (70%) of the green pigments by the ice-cold acetone precipitation procedure; polar lipids are precipitated in cold acetone, while the neutral fraction, which contains the pigments, remains in solution. All lipids present in the total lipid extract (see lane 1 panel "a") are still observable in the polar fraction and, interestingly, it can be noted that after this procedure, CL appears to be one of the main (30%) polar lipids precipitated by acetone. Therefore, the lipid samples obtained after the ice-cold acetone precipitation were suitable for both polar lipid identification by mass spectrometric analyses and CL isolation.

The purity of the isolated CL (lane 2 of Fig. 1b) was checked by re-chromatography, by using a different elution solvent; re-chromatography of CL band (lane 3) revealed the presence of another phospholipid, whose Rf corresponded to that of the phosphatidic acid (PA) authentic standard. The PA amount was comparable to that of CL, and so, this latter represents about 15% of the total polar lipid extract obtained by precipitation with ice-cold acetone.

Further insights into the lipid composition of BBY and PSII dimer fractions were gained by ESI-MS analyses, described in detail in the following.

The low-resolution ESI-MS analyses (negative ions) reported in Fig. 2 confirm the results obtained by chromatographic analyses. The panels "a" and "b" of the figure refer to the BBY and PSII dimer lipids, respectively. In the two ESI-MS spectra, it can be observed that the same lipid components are present, even if the relative intensities of their signals vary, due to the acetone wash procedure. A family of molecular ions $[M–H]^-$ corresponding to PA, different in the saturation degree, can be observed at 667.6 *m/z*; this latter can be attributed to PA (34:4) (calculated mass 667.4 *m/z*) and could correspond to fragments or product ions produced during the MS analysis from other phospholipids; furthermore, the group of peaks centred at 693.9 *m/z* can also be assigned to a family of PA negative ions, having two C18 acyl chains, possibly arising from CL fragmentations. The cluster centred at 723.0 *m/z* can be attributed to the bicharged molecular ions of CLs ($[M–2H]^{2-}$) having four C18 chains and variable saturation degrees; in particular, the signal at 723.0 *m/z* can be due to a CL with eight total unsaturations (calculated mass 723.5 *m/z*). The intense signals at 741.8 *m/z* and 815.3 *m/z* come from the loss of one proton of PG (34:4) (calculated mass 741.5 *m/z*) and sulfoquinovosyldiacylglycerol SQDG (34:3) (calculated mass 815.5 *m/z*) respectively and, finally, the signals at 833.4 and 837.4 *m/z* can be attributed to molecular ions $[M–H]^-$ corresponding to phosphatidylinositol PI (34:2) (calculated mass 833.5 *m/z*) and PI (34:0) (calculated mass 837.5 *m/z*), respectively.

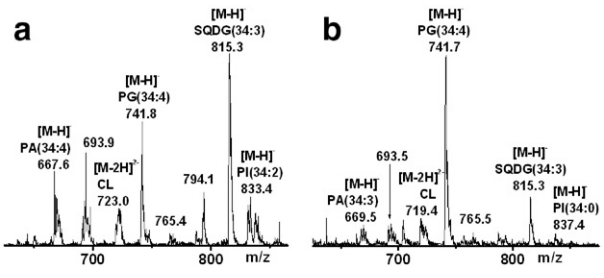

Fig. 2. Low-resolution ESI-MS spectra. ESI-MS (negative ions) of the BBY lipid extract (panel "a") and of the PSII dimer lipid extract (panel "b"), obtained from spinach leaves. Before MS analyses the extracts were incubated with ice cold acetone to remove most of green pigments (see details in Materials and methods).

The presence of PG (34:4) has been considered important for the structural stabilization of the PSII dimers [29–32] especially when it contains trans-hexadecanoic fatty acid (C16:13tr) [4,33]: this fatty acid composition is consistent with the PG (34:4) corresponding to the 741.8 *m/z* peak present in Fig. 2a and b. Moreover, the recent crystal structure of PSII at 3.0 Å resolution reported by Loll et al. [34] evidenced the presence of one PG molecule within the protein scaffold at the interface between the D1 and the CP43 subunits [4,33,35], highlighting the importance of this lipid with respect to protein structural stability. On the other hand, the PG presence is required for dimerization of LHCI and trimerization of LHCII proteins [36–41] in which, according to the recent crystal structure of pea LHCII trimers, a PG molecule has been found at the monomer–monomer interface [36].

From Fig. 2 it can also be observed that in the PSII dimer extract, CL brings about the cluster of molecular ions $[M–2H]^{2-}$ at 719.4 *m/z*, suggesting that the species with all C18 chains and twelve total unsaturations (calculated mass 719.5 *m/z*) could be more strictly related to the protein complex.

It is noteworthy that CL presents all C18 acyl chains, while the other lipids present in the extracts have both C16 and C18 chains; however it can be noted that a low intensity signal at 765.4 *m/z* is present in the spectra of both BBY and PSII dimer lipid extracts and can be attributed to a PG (36:6) molecular ion $[M–H]^-$ (calculated mass 765.5 *m/z*), which can be considered as the monomeric counterpart of the observed CLs. This experimental evidence suggests not only that the PG (34:4) in the lipid extract is not the precursor of the observed CL, but also that CL has characteristics which are specific and different from the other phospholipids.

To better characterize the families of CL peaks present in the low-resolution spectra of Fig. 2, high resolution ESI-MS analysis was performed and the results are reported in Fig. 3. In panel "a" of the figure, the CL band shows three clusters of peaks at 669.47, 693.47 and 721.47 *m/z* in the range 600–800 *m/z*. The cluster at 669.47 *m/z* corresponds to PA (34:3) molecular ion $[M–H]^-$ (calculated mass 669.45 *m/z*), in agreement with TLC analysis previously reported in Fig. 1. Although PA is usually absent in the lipid extract of animal tissues, it is present in significant amount in those of plants, as a product of phospholipase D; its presence in plant extracts has been interpreted as either an instrumental error due to experimental conditions or as the consequence of plant specific cellular responses induced by wounding or osmotic stress conditions [42–47].

Cluster at 721.47 *m/z* ($[M–2H]^{2-}$) is to be attributed to CLs, while signals centred at 693.47 *m/z* could be due to a family of PA negative ions ($[M–H]^-$) resulting from the fragmentation of

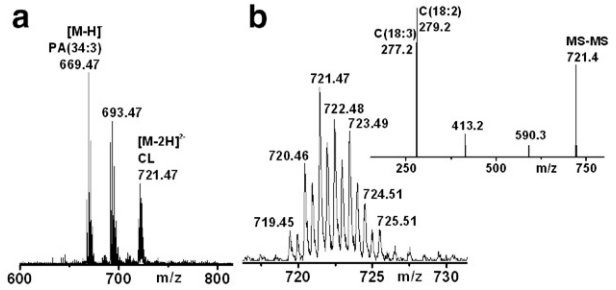

Fig. 3. High resolution ESI-MS spectra. Panel "a": ESI-MS (negative ions) of the CL extract obtained from spinach BBY fragments. Panel "b": ESI-MS spectrum (negative ions) of CL molecular species: this figure is a detail of panel "a"; the inset at the top shows the daughter fragments of the peak at 721.4 *m*/*z* (MS-MS analysis).

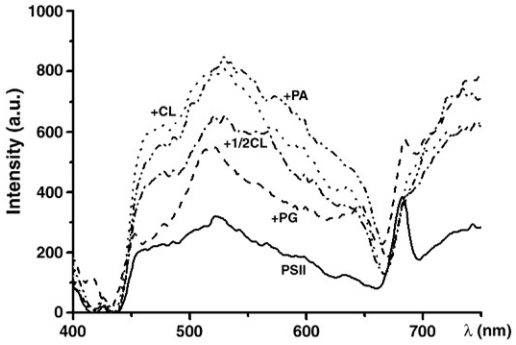

Fig. 4. RLS spectra of PSII monomers in presence or absence of lipids, obtained by subtracting controls and normalizing the minimum scattering value to zero. Measures performed at 25 °C.

the CLs (for PA (36:5) calculated mass is 693.45 *m*/*z*). High resolution MS analysis (panel "b" Fig. 3) showed that the 721.47 *m*/*z* cluster is composed of a family of bicharged CLs whose isotope peaks are separated by about 0.5 *m*/*z*. Therefore, the peaks at 719.45, 720.46, 721.47, 722.48, 723.49 and 724.51 *m*/*z* are attributable to five CLs with different acyl chain saturation degrees. As an example, the most intense peak at 721.47 *m*/*z* is due to a doubly charged CL having four C18 chains and ten total unsaturations (calculated mass 721.46 *m*/*z*).

In order to gain more information about the chains of the analyzed CLs, daughter-fragment spectra (MS-MS analysis) of these molecular species were obtained (see inset panel "b"). The MS-MS spectrum of the molecular species $[M–2H]^{2-}$ corresponding to 721.47 *m*/*z* shows two negative ions $[M–H]^{-}$ at 277.2 and 279.2 *m*/*z*, which represent C18:3 and C18:2 chains, thus confirming the previous assertion that the ion at 721.47 *m*/*z* has ten total unsaturations and that this CL species could be indicated as $[(C18:3)_2/(C18:2)_2\text{-}2H]^{2-}$CL. The other daughter fragments, obtained by breaking the ion 721.4 *m*/*z*, can be also adequately explained but are not of particular importance for our purpose.

Similar considerations can be made on the MS-MS spectra of other doubly charged CL molecular ions $[M–2H]^{2-}$ at 719.45, 720.46, 722.48 and 723.49 *m*/*z*, whose spectra are not shown (see supplementary material) and can be attributed to CLs with all C18 chains and twelve, eleven, nine and eight unsaturations, respectively. Heterogeneity in the CL molecular species has been already found in mitochondrial and in *R. sphaeroides* membranes [48]. Moreover, the presence of CL carrying a significant higher proportion of C18 acyl chains has been reported for mitochondrial membranes [48]. Thanks to these structural peculiarities CL seems to have a particular protein affinity and a propensity to create contacts between different units in the quaternary structure of protein systems, playing, therefore, a functional and a structural role with respect to several membrane proteins. Crystallographic studies on cytochrome *c* oxidase and on the bacterial RC, in fact, have evidenced that CL improves the interactions between different units in these protein complexes [18].

In order to study the influence of CL on PSII structural organization and activity, RLS, fluorescence and OER measurements on PSII monomers were carried out at 25 °C. To verify the specificity of CL, these experiments were extended to PG, in consideration of its abundance in the PSII dimer lipid extract and its importance in the PSII dimerization processes [4], and to PA, which was not expected to give specific interactions with the protein complexes. Fig. 4 shows the RLS spectra of PSII complex in presence or absence of lipids; the spectra, each average of three replicates, were obtained by subtracting controls (see supplementary material, Fig. 7) and normalizing the minimum scattering value to zero.

RLS is very sensitive and selective in probing the aggregation processes which involve chromophores as the higher the coupling among chromophores the higher the intensity of the signal is [49,50]. The increment of the RLS peak intensity recorded for PSII monomers in presence of PG or CL suggests that an aggregation process is favoured by phospholipids, and in particular by CL [4,51,52]. Moreover, since CL can be considered as the dimeric counterpart of PG and in the experiments reported CL showed signals which were almost two times as intense compared to PG, when used at the same molarity, the effect of halving CL molarity was also studied (see trace labelled as 1/2CL in Fig. 4). As a result, CL at half the molarity caused an increment of the intensity of PSII aggregate scattering, which was lower than that of PSII in presence of CL but yet higher than that in presence of PG. Surprisingly, the

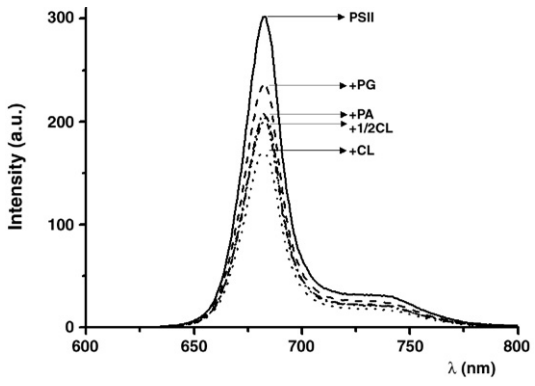

Fig. 5. Fluorescence emission spectra of PSII monomers in presence or absence of lipids. Measures performed at 25 °C.

addition of PA at the same concentration of CL, resulted in a similar aggregation effect.

The fluorescence emission spectra (reported in Fig. 5) show a characteristic band at 683 nm and a shoulder at 740 nm, which most probably originated from a vibrational sublevel, as reported in literature [53–55]. A decrease in fluorescence intensity is observed when exogenous PG or CL is added to PSII complexes; the intensity decrement is greater for CL, even at half the molarity. Since chlorophyll *a* fluorescence quenching has been reported to take place exclusively in the presence of aggregates, as in the case of LHCII complexes [56], these results could again suggest that phospholipids, in particular CL, enhance an aggregation process in PSII moieties. In these experiments the effect of PA was similar to that obtained with 1/2CL.

The effect of phospholipids on the functionality of PSII complexes was investigated by means of OER measurements, observing the consequence of adding exogenous PG or CL to PSII monomers before thermal inactivation. Oxygen evolution is one of the most heat-sensitive processes in photosynthesis: previously reported results indicate that the 33-kDa extrinsic subunit of PSII, could be the primary cause of thermal inactivation. In fact, at moderately high temperature (40 °C) the oxygen evolution inactivation mechanism could depend upon the dissociation of this 33-kDa polypeptide from its own binding site. Conformational changes in the secondary structure of the 33-kDa subunit could be responsible of this dissociation and affect the loss in oxygen evolution efficiency [57–64]. Fig. 6 reports the PSII monomer OER percentage preservation after 2 min of incubation at 40 °C. The values are normalized with respect to the OER at zero time; all the reported data represent mean values±standard deviation obtained from three replicates. The presence of exogenous phospholipids seems to stabilize PSII complexes with respect to thermal inactivation; in particular PSII activity appears to be better preserved in presence of CL, even at half the molarity.

Regarding PA, results obtained by OER measurements are in contrast with RLS and fluorescence data. Although PA seemed to have a role in the aggregation of PSII complexes, its presence resulted in inactivation of the PSII oxygen evolution rate during the measuring time. Therefore, it can be concluded that the presence of PA could result in PSII unspecific and non-functional aggregation.

The greater stability of PSII against thermal inactivation in presence of CL could be related to the formation of active PSII aggregates, in accordance with higher oxygen evolution rates reported for PSII dimers with respect to monomers [7] and for PSII aggregates obtained in lipid vesicles [29,65]. Data in literature show that CL binds preferentially at monomer interfaces of oligomeric assemblies and at subunit interfaces of multisubunit complexes [66] as reported for homodimeric bovine cytochrome *c* oxidase [67] and for the trimeric formate dehydrogenase N [68].

Since the 3-Å structure of PSII dimers reported by Loll et al. [34] did not show the presence of buried or tightly bound CLs it can be supposed that CL could be located at the interface between PSII complexes, stabilizing the formation of supercomplexes.

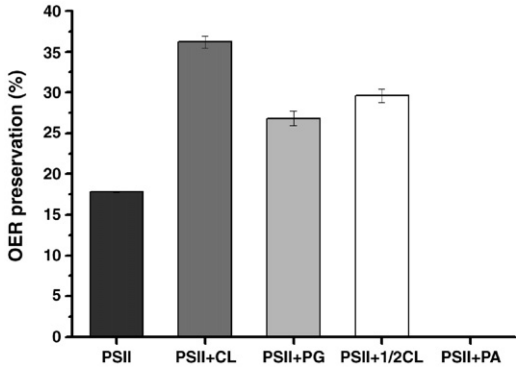

Fig. 6. PSII monomer OER percentage preservation after 2 min of incubation at 40 °C, in absence or in presence of lipids.

The overall experimental evidence presented in this paper seems to concordantly indicate the peculiarity of the role played by CL and PG in regulating PSII complex aggregation processes and activity. The combination of TLC and low and high resolution ESI-MS analyses allowed the characterization of the lipid profiles of BBYs and PSII dimers, evidencing in particular that in these membrane complexes CL is present as species having four C18 unsaturated acyl chains and variable saturation degrees. The peculiar characteristics of this lipid in influencing interaction between different monomeric units of PSII were convincingly evidenced by fluorescence and RLS measurements. The increased resistance of the photosynthetic protein to thermal denaturation in the presence of added CL and PG represented further evidence of the role played by these lipids in promoting PSII aggregation and stabilization.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2007.03.024.

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